UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A130 634
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) DIFFERENTIATION OF BACILLUS ANTHRACIS AND OTHER BACILLUS SPECIES BY USE OF LECTINS	5. TYPE OF REPORT & PERIOD COVERED Interim
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) HUGH B. COLE, JOHN W. EZZELL, JR., KENNETH F. KELLER, AND RONALD J. DOYLE*	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Army Medical Research Institute of Infectious Diseases SGRD-UIB-A Fort Detrick, Frederick, MD 21701	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 871-BB-149
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Office of the Surgeon General Department of the Army, Washington, D.C. 20314	12. REPORT DATE 18 July 1983
	13. NUMBER OF PAGES 27 pages
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) UNCLASSIFIED
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Information regarding reprints not available at this time. To be published in the Journal of Clinical Microbiology.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Bacillus anthracis, lectins

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Bacillus anthracis was agglutinated by several lectins, including those from Griffonia simplicifolia, Glycine max, Abrus precatorius, and Ricinus communis. Some strains of Bacillus cereus var. mycoides (B. mycoides) were strongly reactive with the lectin from Helix pomatia and weakly reactive with the Glycine max lectin. The differential interactions between Bacillus species and lectins afforded a means of distinguishing B. anthracis from other bacilli. B. cereus strains exhibited heterogeneity with respect to agglutination patterns by lectins, but could readily be differentiated from B. anthracis and the related

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

B. mycoides. Spores of B. anthracis and B. mycoides retained lectin receptors, although the heating of spores or vegetative cells at 100°C resulted in a decrease in their ability to be specifically agglutinated. Fluorescein-conjugated lectin of Glycine max stained vegetative cells of B. anthracis uniformly suggesting that the distribution of lectin receptors was continuous over the entire cellular surface. B. anthracis cells grown under conditions to promote the production of capsular poly (D-glutamyl peptide) were also readily agglutinated by the lectins, suggesting that the lectin reactive sites penetrate the polypeptide layer. Trypsin, subtilisin, lysozyme and mutanolysin did not modify the reactivity of B. anthracis with the Glycine max agglutinin, although the same enzymes markedly diminished the interaction between the lectin and B. mycoides. Because the lectins which interact with B. anthracis are specific for α-D-galactose or 2-acetamido-2-deoxy-α-D galactose residues, it is likely that the bacteria possess cell surface polymers which contain these sugars. Lectins may prove useful in the laboratory identification of closely related Bacillus species.

Differentiation of *Bacillus anthracis* and other *Bacillus* species by use of Lectins

Hugh B. Cole[1], John W. Ezzell, Jr.[2],
Kenneth F. Keller[1], and Ponald J. Doyle*[1]

Department of Microbiology and Immunology,
University of Louisville Health Sciences Center,
Louisville, KY 40292

and

[2]Division of Bacteriology,
U.S. Army Medical Research Institute of Infectious Diseases,
Ft. Detrick, MD 21701

Short title: Lectin - *B. anthracis* interaction

*Correspondent author (502) 588-5350

ABSTRACT

Bacillus anthracis was agglutinated by several lectins, including those from *Griffonia simplicifolia*, *Glycine max*, *Abrus precatorius*, and *Ricinus communis*. Some strains of *Bacillus cereus var. mycoides* (*B. mycoides*) were strongly reactive with the lectin from *Helix pomatia* and weakly reactive with the *Glycine max* lectin. The differential interactions between *Bacillus species* and lectins afforded a means of distinguishing *B. anthracis* from other bacilli. *B. cereus* strains exhibited heterogeneity with respect to agglutination patterns by lectins, but could readily be differentiated from *B. anthracis* and the related *B. mycoides*. Spores of *B. anthracis* and *B. mycoides* retained lectin receptors, although the heating of spores or vegetative cells at 100°C resulted in a decrease in their ability to be specifically agglutinated. Fluorescein-conjugated lectin of *Glycine max* stained vegetative cells of *B. anthracis* uniformly suggesting that the distribution of lectin receptors was continuous over the entire cellular surface. *B. anthracis* cells grown under conditions to promote the production of capsular poly(D-glutamyl peptide) were also readily agglutinated by the lectins, suggesting that the lectin reactive sites penetrate the polypeptide layer. Trypsin, subtilisin, lysozyme and mutanolysin did not modify the reactivity of *B. anthracis* with the *Glycine max* agglutinin, although the same enzymes markedly diminished the interaction between the lectin and *B. mycoides*. Because the lectins which interact with *B. anthracis* are specific for α-D-galactose or 2-acetamido-2-deoxy-α-D galactose residues, it is likely that the bacteria possess cell surface polymers which contain these sugars. Lectins may prove useful in the laboratory identification of closely related *Bacillus* species.

INTRODUCTION

Most species of Bacillus are saprophytic and are widely distributed in nature, particularly in soils. One organism, B. anthracis, is an important pathogen in man and cattle and may lead to a serious disease called anthrax. The laboratory identification of members of the genus Bacillus may involve biochemical reactions, immunofluorescence, bacteriophage typing, production of capsule, analysis composition of lipids and determination of nucleic acid homologies (reviewed by Berkeley and Goodfellow, ref 1). There are close relationships between B. anthracis, B. cereus, B. mycoides, and B. thuringiensis in terms of antigenic structures of surface components (5,11,16,18,19,21), metabolism (14,17,22,23) and DNA-DNA homologies (15,31,32). Serologic methods have generally been unsuccessful in identifying B. anthracis (5,11,16). Moreover, bacteriophage typing is not absolutely specific as other bacilli may adsorb B. anthracis bacteriophage (2,5). Studies have concluded that there is no single criterion, including pathogenicity, that separates B. anthracis, B. cereus, B. mycoides, and B. thuringiensis (26).

We have noted that lectins are convenient reagents for the study of cell surfaces of bacilli (4,30). The glucosylated cell wall teichoic acid of B. subtilis 168 can be purified using affinity chromatography on concanavalin A-Sepharose columns (7). Furthermore, the distribution of wall teichoic acids on the B. subtilis cell surface can be monitored by use of fluorescent concanavalin A (8). Because B. anthracis is known to possess a galactose-containing polysaccharide on its cell envelope (3,24) it was reasoned that galactose-binding lectins may be agents which could selectively agglutinate the bacterium. In this report, we describe procedures which enable the rapid differentiation of B. anthracis from other bacilli. The methods employ galactose-binding lectins and can be completed within a few minutes.

MATERIALS AND METHODS

Reagents and Chemicals

All lectins and agglutinins, including fluorescein-labeled soybean agglutinin, were supplied by E-Y Laboratories (San Mateo , CA) (TABLE 1). The lectins were affinity purified, except for SRA (Sarothamnus scoparius), which was an ammonium sulfate precipitate. Calcium chloride was obtained from J.T. Baker Chemical Co. (Phillipsburg, N.J.). Reagent manganous chloride and urea were obtained from Fisher Scientific Co. (Fairlawn, N.J.). Reagent grade sucrose, sodium dodecyl sulfate (SDS), trypsin, subtilisin, succinic anhydride and lysozyme were products of Sigma Chemical Co., St. Louis. Complex media were obtained from Difco (Detroit, MI) or from Baltimore Biological Laboratories (Cockeysville, MD). Mutanolysin (36) was a gift from Dr. K. Yokogawa, Dainippon, Ltd., Osaka.

Organisms and culture conditions

Sources of strains of Bacillus used are listed in TABLE 2. All strains were maintained on AK sporulation agar (BBL, Cockeysville, MD), except for B. globisporus, which was maintained on tryptose blood agar base (TBAB; Difco Laboratories, Detroit, MI). Cells and spores were stored at 4° C prior to transfer to new slants or media. For agglutination assays, most cells were obtained from overnight growth at 37°C on TBAB agar plates, whereas B. globisporus and B. mycoides were cultured at room temperature prior to harvesting. Cells were recovered by use of a wetted cotton swab and suspended in PBS (40 mM sodium phosphate, 150 mM sodium chloride, 0.1 mg/mL sodium azide , pH 7.3).

Spore growth and preparation

Sporulation was accomplished by a modification of the method used by Eisenstadt and Silver (9). Inocula were taken from TBAB plates and suspended in tryptic soy broth (TSB; Difco Laboratories), supplemented with 100 μM calcium

chloride and 10 μM manganous chloride. Cells were vigorously shaken for 64 hr at 37°C. Spores were washed twice in PBS, then further purified by sedimenting twice in 55% sucrose. The enriched spores were then suspended in PBS to an optical density of 0.6 ± 0.1 at 450 nm (1-cm pathlength) and incubated with mutanolysin (50 μg/mL final concentration in PBS) or lysozyme (50 μg/mL) for 17 ± 2 hr at 37°C. Spores were then washed twice by centrifugation and suspension in PBS. Preparations were examined by Gram stain and by phase contrast microscopy for rod-shaped cells. Only spore preparations judged to be free of intact cells were used in agglutination assays.

Agglutination test procedures

Procedures for agglutination were adapted from the methods used by Schaefer et al. (30) for Neisseria. Both vegetative cell and spore suspensions were tested in the same manner. Agglutination tests were carried out on Boerner microtiter plates (Curtin Matheson Scientific, Inc., Cincinnati, OH). Lectins were diluted in PBS to a concentration of 200 μg/mL and stored at 4°C. Test wells were set up opposite to control wells for direct test-control comparisons. In each test well, 50 μL of cell suspension was added to 50 μL of lectin. In one control well, 50 μL of buffer was added to 50 μL lectin to detect any false-positives due to a precipitation reaction between lectin and buffer. In the other control well, 50 μL cell suspension was mixed c̄ 50 μL buffer. Plates were then shaken on a Tektator V rotary shaker for 10 min at 150 rpm. It was important not to permit the cells to incubate with the lectins for extended time periods, e.g. > 1 hr, as a loss of specificity was observed. Plates were examined for evidence of agglutination reaction under an Olympus VMT stereo microscope. Occasionally, cells exhibited autoagglutination in PBS. In most cases, these autoagglutinations were not significant enough to bias the lectin agglutination readings.

Fluorescein labeling of cells and spores

Fluorescein-labeled SBA (Fl-SBA, 400 µg/mL in PBS) was mixed with an equal volume of cells (usually 100 µL) or spores. The suspensions were incubated at room temperature for 10-15 min with gentle shaking. The suspensions were then washed twice in PBS to remove unbound lectin. Samples were finally dried on microscope slides. Specimens were observed by fluorescence microscopy (Carl Zeiss, Inc., New York). Photographs were taken with a Nikon FM with a Nikormat model 2 microscope adapter (Nikon In., Garden City, NY) using Kodak ASA 400 color print film (Eastman Kodak Co., Rochester, NY).

RESULTS

Interaction between Bacillus species and lectins

In order to compare agglutination patterns, the Bacillus species were arbitrarily placed into API groups. The API groupings for Bacillus depend on metabolic activities (22,23) and are useful in establishing taxonomic relationships between closely related species. Group I included B. anthracis, B. cereus, B. mycoides, and B. thuringiensis (22). TABLE 3 shows interaction of the bacilli suspensions with purified lectins (see TABLE 1 for description of lectins). All strains of B. anthracis were agglutinated by RCA-I, RCA-II, APA, GSA-I, and SBA. Similar reactivities were exhibited by B. mycoides, but these species were also agglutinated by HPA. The lectins which agglutinated B. anthracis and B. mycoides were capable of interating with D-galactose or 2-acetamido-2-deoxy-D-galactose (TABLE 1). Some lectins, however, with similar carbohydrate-binding specificies, were incapable of agglutinating the bacilli (TABLE 3).

For B. cereus strains, great heterogeneity was observed in terms of interactions with the lectins (TABLE 3). Several B. cereus strains were unreactive with all of the lectins. A general pattern, reflective of all B. cereus strains, was not apparent. None of the B. cereus strains were agglutinated by SBA or APA. B. thuringiensis strains were also generally refractory to lectins. This is significant as B. thuringiensis strains are generally difficult to differentiate from B. anthracis.

Representative species of other Bacillus API groups were found not to readily agglutinate with lectins (TABLE 4). Only B. sphaericus, B. subtilis 168 and B. amyloliquefaciens were agglutinable with Con A. Probably the cell receptor responsible for interaction with Con A was α-D-glucosylated teichoic acids (6, 7, 8). Weak agglutination of B. subtilis strains 168 and W23 by

LPA was observed, possibly due to specific interaction between the lectin and glycerol or ribitol teichoic acids (28).

Bacillus spores and lectins

Members of the genus Bacillus can undergo metabolic changes leading to the formation of endospores. The spores are generally considered to possess internal cell wall components surrounded by multiple coats of protein (20). During the vegetative cell to spore transition, considerable surface modification must occur, but is unknown whether the spores retain lectin-reactive sites, or even whether there are new and different sites synthesized. Lectin agglutination tests for B. anthracis and other bacilli would be greatly strengthened if the spores retained their lectin receptors. We purified spores of several Bacillus species by density centrifugation in sucrose and by digestion of intact cells with lysozyme and mutanolysin (36) (in other experiments we have found that mutanolysin is a useful enzyme for the dissolution of walls of API Group I bacilli, G. Zipperle, J. Ezzell, and R.J. Doyle, submitted). Purified spores were then mixed with lectins (TABLE 5). The results provide evidence to suggest that B. anthracis and B. mycoides can be distinguished by lectins. In fact, spores and vegetative cells of both of these species appear to be agglutinated by the same lectins (TABLE 3). The HAA lectin was able to weakly agglutinate several spores from B. cereus strains but not the respective vegetative cells. Furthermore, MPA, UEA-II and Con A were able to agglutinate some spores but no vegetative cells. B. subtilis 168 vegetative cells and spores were agglutinated by Con A. The results support the view that lectins can also be used as selective agglutinating reagents for bacterial spores.

Vegetative cells and spores of several strains of B. anthracis and B. mycoides were titrated with SBA, GSA-I, and HPA. It was found that in general, B. anthracis vegetative cells could more readily bind SBA than B. mycoides cells

(TABLE 6). When the agglutinations of spores were compared to vegetative cells, it was observed that the spores tended to interact less strongly with lectins. When either cells or spores were heated to 100°C for 15 min and cooled, a higher concentration of lectin was usually required to elicit agglutination. The heating of cells or spores apparently results in loss or modification of lectin-binding sites.

Distribution of lectin binding sites on B. anthracis

In previous studies it was shown that fluorescein-labeled Con A bound over the entire surface of B. subtilis, although the lectin may have been more concentrated at septa (8). Lectin receptor sites may possibly be found on cell poles, septa and cylinders of bacilli. Washed cells and spores of B. anthracis ATCC 11966 were interacted with Fl-SBA and examined by fluorescence microscopy. The results (Figs. 1 and 2) reveal that the lectin tends to bind evenly over all parts of the vegetative cells. Moreover, the results also confirm the observation that spores of B. anthracis interact with the lectin.

Interaction of bacilli with anti-lipoteichoic acid antibody

Results showing that both vegetative cells and spores of B. anthracis and B. mycoides possessed lectin-binding sites suggested that even membrane components, such as lipoteichoic acids, may be surface-exposed. Van Driel et al. (34) observed that although certain Lactobacillus did not possess cell wall teichoic acids, the bacteria could still be agglutinated by anti-poly(glycerolphosphate) antisera. These results were presented as evidence to show that membrane lipoteichoic acids (a poly-glycerolphosphate) can be prominent surface antigens. We have found that an antibody preparation (33) directed against the lipoteichoic acid of B. subtilis gtaB290 will agglutinate cells and spores of B. anthracis, B. mycoides, and B. subtilis 168 (TABLE 7). The agglutination occurs even though the antiserum had been adsorbed with cell walls of B. subtilis W23, a procedure employed to eliminate antipeptidoglycan antibodies. Cell walls of B. anthracis

and B. mycoides strains were found lacking in either poly (glycerolphosphate) or poly(ribitolphosphate) (G. Zipperle, J. Ezzell, and R.J. Doyle, unpublished results). The results suggest that poly(glycerolphosphate) is a surface component in vegetative cells and spores of B. anthracis and B. mycoides.

Removal of lectin receptors from B. anthracis and B. mycoides

B. anthracis and B. mycoides were subjected to several kinds of extractions or enzyme treatments in order to modify lectin receptor sites such that one organism may be more readily differentiated from the other by either SBA or HPA. The cells were treated with protein extractants (0.1% sodium dodecylsulfate) and proteases (trypsin and subtilisin). If lectin receptors were removed or modified by the treatments, then the amounts of lectins required for agglutination may be changed. The results (TABLE 8) show that lysozyme, mutanolysin, trypsin, and subtilisin destroyed or weakened the agglutinability of B. mycoides ATCC 6462 by SBA, whereas HPA receptors remained intact. In addition, 8 M urea was also effective in rendering B. mycoides insensitive to SBA. Treatment of B. anthracis, in contrast, by the same enzymes or extractants did not greatly modify reactivity with either SBA or HPA. One reagent, succinic anhydride, designed to increase the overall negative surface charge, did not alter the binding of either B. anthracis or B. mycoides with the two lectins. Overall, the results appear to reveal that the surface of B. mycoides is less resistant than B. anthracis to chemical or protease challenge.

DISCUSSION

Several factors may be involved in the interaction between bacterial cell surfaces and lectins. Not only must an organism possess the proper carbohydrate determinants on its surface, but other factors such as lectin molecular weight, hydrophobic group stabilization, hydrogen ion concentration and ionic strength are also important. Results from this study show that several factors may be involved in lectin-*Bacillus* complex formation.

When *Bacillus* species were interacted with purified lectins of differing specificities, it was observed that several of the proteins could agglutinate *B. anthracis* and *B. mycoides* strains. These lectins were generally of a specificity for D-galactose or 2-acetamido-2-deoxy-D-galactose (GalNAc) and included SBA, GSA-I, RCA-I and II, and APA (TABLE 3). Another lectin, HPA, also specific for GalNAc and D-Gal, agglutinated *B. mycoides*, but not *B. anthracis* thereby affording a means of distinguishing *B. anthracis* from *B. mycoides*. It is surprising that lectins such as BPA, MPA, HAA, LBA and others, although readily reactive with Gal or GalNAc groups (12), would agglutinate neither *B. anthracis* nor *B. mycoides*. The results suggest a rapid means of identifying *B. anthracis* from a colony or pure culture. Agglutination by SBA, the non-toxic soybean agglutinin, identifies the cells as either *B. anthracis* or *B. mycoides* and the HPA lectin specifically agglutinates the latter bacterium. Moreover, spores can also be identified by the same means (TABLE 5).

The composition of the polymer(s) or cell surface component(s) responsible for interacting with the lectins is unknown. Mester *et al.* reported that *B. anthracis* possessed a polymer composed of Gal, acetylated galactose, and 2-amino-2-deoxy-D lucose (24). This polymer was poorly immunogenic in rabbits (3,13) and may n be prominent surface antigen of the organism. The diagnostic value of the po mer may have therefore been overlooked. It is possible that the reactive lectins were able to interact with this polymer in *B. anthracis* and its close taxonomic species, *B. mycoides*. Because the molecular weight of SBA is

120,000 (12) and the molecular weight of HPA is 26,000 (12) it is assumed that the tertiary structures of the lectins govern their ability to bind to potential receptors on cell surfaces. Steric factors may also be involved in the inhibition of γ-phage binding to B. anthracis by WGA ( ), even though WGA does not agglutinate the bacteria (TABLE 3).

The fact that spores retained lectin-binding sites can possibly be explained. The spores may have retained the lectin receptors in an unmodified form, and the receptors could have penetrated the spore coats or could have been components of the spore coats. Conversely, the spores may contain completely different lectin receptors, but of similar composition, and were therefore capable of interacting with the lectins. Support for this view comes from the observations that several B. cereus strains expressed different lectin receptors on spores and vegetative cells (TABLES 3 and 5). For example, cells of B. cereus T were refractory to agglutination by WGA, HAA and GSA-II, whereas spores were agglutinated by these lectins. It is known that spores and vegetative cells of several Bacillus species possess common antigens (,). These antigens may, in certain cases, be the lectin receptors. Finally, it must also be considered that the spores were not completely freed of vegetative cells or cell structures.

The results also reveal the heterogeneity of B. cereus strains. A lectin specific for GlcNAc, WGA, agglutinated only B. cereus strains 4915 and 11778 (TABLE 3). The lectin GSA-I, specific for Gal and GalNAc residues, agglutinated only B. cereus strains 11778, E14578 and 7064. B. cereus strain T was agglutinated by LPA. A general pattern of reactivity was not found for B. cereus, although the results clearly distinguish B. cereus from B. anthracis and B. mycoides.

The observation that anti-poly(glycerolphosphate) will agglutinate B. anthracis and B. mycoides cells and spores may be explained several ways. The cells and spores may contain lipoteichoic acids which penetrate their surfaces, thereby enabling the antibodies to contact their complementary receptors. In contrast,

the antibody preparation may not have been monospecific, although care was taken in antigen preparation (33) and in adsorption of anti-peptidoglycan antibodies (TABLE 7).

When cells or spores were boiled in PBS prior to interaction with lectin, it was found that more lectin was usually required to elicit agglutination (TABLE 6). These results suggest that the heat treatment may have extracted some of the lectin receptors. Another explanation is that heat treatment changed the conformation or distribution of the receptors, although this does not appear likely. The observations that proteases and chaotropic agents do not markedly modify reactivity of B. anthracis with SBA suggests that the lectin-binding sites on the cells are not protein, nor are they necessarily associated with surface protein. The receptors must contain Gal or GalNAc, but it is unlikely that these carbohydrates are covalently bound to protein, as glycoproteins in bacteria are rare. The loss of agglutinability by SBA when B. mycoides was treated with heat, detergents or enzymes may suggest that the SBA receptors were removed or extracted. The retention of HPA receptors by B. mycoides, in contrast, following the same treatments suggests that the HPA receptors and the SBA receptors are distinct molecules, although both receptors probably contain D-Gal or D-GalNAc.

We believe that lectins may have importance in the taxonomic classification of Bacillus species. The results of this paper provide evidence which show that B. anthracis and B. mycoides can be distinguished from each other and from other bacilli by use of only two lectins. Because lectins are monoclonal proteins and ~~because~~ they possess a spectrum of specificities and molecular weights, ~~it is to be expected~~ we expect that they will be excellent provide substantial tools for diagnostic and taxonomic studies.

ACKNOWLEDGMENTS

This work was supported in part by Contract DAMD17 81C-1028 from the U.S. Army. F. Nedjat-Haiem provided expert assistance with some of the experiments. The ~~assiduous~~ assistance of Suzanne Langemeier in photography instruction is gratefully recognized.

LITERATURE CITED

1. Berkeley, R.C.W. and M. Goodfellow . 1981. The aerobic endospore forming bacteria: classification and identification. Academic Press. New York, NY.

2. Buck, C.A., R.L. Anacker, F.S. Newman, and A. Eisenstark. 1963. Phage isolated from lysogenic Bacillus anthracis. J. Bacteriol. 85:1423-1430.

3. Cave-Browne-Cave, J.E., E.S.J. Fry, H.S. El Khadem and H.N. Rydon. 1954. Two serologically active polysaccharides from Bacillus anthracis. J. Chem. Soc. 3866-3874.

4. Davidson, S.K., K.F. Keller and R.J. Doyle. 1982. Differentiation of coagulase-positive and coagulase-negative staphylococci by lectins and plant agglutinins . J. Clin. Microbiol. 15:547-553.

5. Dowdle, W.R. and P.A. Hansen. 1961. A phage-fluorescent antiphage staining system for Bacillus anthracis. J. Infect. Dis. 108:125-135.

6. Doyle, R.J., and D.C. Birdsell. 1972. Interaction of concanavalin A with the cell wall of Bacillus subtilis. J. Bacteriol. 109:652-658.

7. Doyle, R.J., D.C. Birdsell and F.E. Young. 1973. Isolation of the teichoic acid of Bacillus subtilis 168 by affinity chromatography. Preparative Biochem. 3:13-18.

8. Doyle, R.J., M.L. McDannel, J.R. Helman and U.N. Streips. 1975. Distribution of teichoic acid in the cell wall of Bacillus subtilis. J. Bacteriol. 122:152-158.

9. Eisenstadt, E. and S. Silver. 1972. Calcium transport during sporulation in Bacillus subtilis. p. 180-186. In H.P. Halvorson, R. Hanson and L.L. Campbell (eds.). Spores V. American Society for Microbiology. Washington, D.C.

10. Feeley, J.C., and C.M. Patton. 1980. Bacillus. pp. 145-149. In E.H. Lennette, A. Balows, W.J. Hausler Jr. and J.P. Truant (eds.), Manual for Clinical Microbiology, 3rd edition, American Society of Microbiology. Washington, D.C.

11. Fluck, R., R. Bohm and D. Strauch. 1977. Fluorescent serological studies on cross reactions between spores of Bacillus anthracis and spores of other aerobic sporeforming bacteria. Zentralbl. Veterinaermed. Reiche. B. 24:497-507.

12. Goldstein, I.J. and C.E. Hayes. 1978. The lectins: carbohydrate-binding proteins of plants and animals. p. 127-340. In R.S. Tipson and D. Horton (eds.), Advances in Carbohydrate Chemistry and Biochemistry. Academic Press, New York, NY.

13. Ivanovics, G. 1940. Das serologische verhalten der abbauprodukte des anthrax-polysaccharide. Zeitschr. Immunitatsforsch. 98:420-426.

14. Kaneda, T. 1968. Fatty acids in the genus Bacillus. II. Similarity in the fatty acid compositions of Bacillus thuringiensis, Bacillus anthracis and Bacillus cereus. J. Bacteriol. 95:2210-2216.

15. Kaneko, T., R. Nozaki and K. Aizawa. 1978. Deoxyribonucleic acid relatedness between Bacillus anthracis, Bacillus cereus and Bacillus thuringiensis. Microbiol. Immunol. 22:639-641.

16. Kim, H.U., and J.M. Goepfert. 1972. Efficacy of a fluorescent-antibody procedure for identifying Bacillus cereus in foods. Applied Microbiol. 24:708-713.

17. Knisely, R.F. 1965. Differential media for the identification of Bacillus anthracis. J. Bacteriol. 90:1778-1783.

18. Lamanna, C., and D. Eisler, 1960. Comparative study of the agglutinogens of the endospores of Bacillus anthracis and Bacillus cereus. J. Bacteriol. 79:435-441.

19. Lamanna, C. and L. Jones. 1961. Antigenic relationship of the endospores of Bacillus cereus-like insect pathogens to Bacillus cereus and Bacillus anthracis. J. Bacteriol. 81:622-625.

20. Leive, L.L. and B.D. Davis. 1980. Cell envelope; Spores. pp. 71-110. In B.D. Davis, R. Dulbecco, H.N. Eisen and H.S. Ginsberg (eds.), Microbiology, 3rd Edition. Harper & Row Publishers, Inc. Hagerstown, MD.

21. Levina, E.N., and L.N. Katz. 1966. A study of Bacillus anthracis and Bacillus cereus antigens with the aid of fluorescent serological and cytochemical methods of investigation. Zh. Mikrobiol. Epidemiol. Immunobiol. 4364: 98-103.

22. Logan, N.A. and R.C.W. Berkeley. 1981. Classification and identification of members of the genus Bacillus using API test. p. 105-140. In R.C.W. Berkeley and M. Goodfellow (eds.), The aerobic endospore-forming bacteria. Academic Press. New York, NY.

23. Logan, N.A., B.J. Capel, J. Melling and R.C.W. Berkeley. 1979. Distinction between emetic and other strains of Bacillus cereus using the API system and numerical methods. FEMS Microbiol. Lett. 5:373-375.

24. Mester, L., E. Moczar, and J. Trefouel. 1962. Sur les groupements terminaux du polysaccharide immunospecifique du Bacillus anthracis. Comp. Rend. Acad. Sci. (Fr) 255:944-945.

25. Norris, J.R. 1962. Bacterial spore antigens: a review. J. Gen. Microbiol. 28:393-408.

26. Pederson, C.S. 1956. Symposium on problems in taxonomy. Bacteriol. Rev. 20:274-276.

27. Phillips, A.P., K.L. Martin and M.G. Broster. 1983. Differentiation between spores of Bacillus anthracis and Bacillus cereus by a quantitative immunofluorescence technique. J. Clin. Microbiol. 17:41-47.

28. Pistole, T.G. 1978. Broad spectrum bacterial agglutinating activity in serum of horseshoe crab, Limulus polyphemus. Devel. Comp. Immunol. 2:65-76.

29. Priest, F.G. 1981. DNA Homology in the enus Bacillus. pp. 33-57. In R.C.W. Berkeley and M. Goodfellow (eds.). The aerobic endospore-forming bacteria. Academic Press. New York, NY.

30. Schaefer, R.L., K.F. Keller, and R.J. Doyle. 1979. Lectins in diagnostic microbiology: use of wheat germ agglutinin for laboratory identification of Neisseria gonorrhoeae. J. Clin. Microbiol. 10:669-672.

31. Seki, T., C. Chung, H. Mikami and Y. Oshima. 1978. Deoxyribonucleic acid homology and taxonomy of the genus Bacillus. Internat. J. System. Bacteriol. 28:182-189.

32. Somerville, H.J., and M.L. Jones. 1972. DNA competition studies within the Bacillus cereus group of bacilli. J. Gen. Microbiol. 73:257-265.

33. Thaniyavarn, S., R.J. Doyle, H.L.T. Mobley and U.N. Streips. 1979. Lipoteichoic acids from D-glucosylation-defective, cell wall mutants of Bacillus subtilis. Carbohydr. Res. 73:203-218.

34. Van Driel, D., A.J. Wicken, M.R. Dickson and K.W. Knox. 1973. Cellular location of the lipoteichoic acids of Lactobacillus fermenti NCTC 6991 and Lactobacillus casei NCTC 6375. J. Ultrastr. Res. 43:483-497.

35. Watanabe, T., and T. Shiomi. 1976. Effect of plant lectins on γ phage receptor sites of Bacillus anthracis. Japan. J. Microbiol. 20:147-149.

36. Yokagawa, K.S. Karvata, S. Nishimura, Y. Ikeda, and Y. Yoshimura. 1974. Mutanolysin, bacteriolytic agent for cariogenic streptococci; partial purification and properties. Antimicrob. Ag. Chemoth. 6:156-165.

TABLE 1. Lectins used to agglutinate *Bacillus* species[a].

Lectin	Specificity[b]
Abrus precatorius (APA)	β-D-Gal > α-D-Gal
Arachis hypogeae (PNA)	D-Gal-β-(1→3) > β-D-GalNH$_2$ = α-D-Gal
Bauhinia purpurea (BPA)	D-GalNAc > D-Gal
Canavalia ensiformis (Con A)	α-D-Man > α-D-Glc > α-D-GlcNAc
Dolichos biflorus (DBA)	α-D-GalNAc > α-D-Gal
Glycine max (SBA)	α-D-GalNAc ≥ β-D-GalNAc >> α-D-Gal
Griffonia simplicifolia (GSA-I)	α-D-Gal > α-D-GalNAc
Griffonia simplicifolia (GSA-II)	α-D-GlcNAc = β-D-GlcNAc
Helix aspersa (HAA)	α-D-GalNAc = α-D-GlcNAc
Helix pomatia (HPA)	α-D-GalNAc > α-D-GlcNAc >> α-D-Gal
Limulus polyphemus (LPA)	sialic acid
Lotus tetragonolobus (Lotus A)	α-L-Fuc = 2-O-Me-D-Fuc
Maclura pomifera (MPA)	α-D-Gal = α-D-GalNAc
Phaseolus limensis (LBA)	α-D-GalNAc > α-D-Gal
Phaseolus vulgaris (PHA-E)	D-GalNAc
Pisum sativum (PEA)	α-D-Man > α-D-Glc > α-D-GlcNAc
Ricinus communis (RCA-I)	β-D-Gal > α-D-Gal
Ricinus communis (RCA-II)	β-D-Gal > β-D-GalNAc
Robina pseudoacacia (RPA)	unkrown, (possibly sialoglycopeptides)
Sarothamnus scoparius (SRA)	α-D-Gal > α-L-Fuc
Solanium tuberosum (STA)	$(\beta\text{-D-GlcNAc})_{2-5}$ > β-D-GlcNAc
Sophora japonica (SJA)	β-D-GalNAc > β-D-Gal
Triticum vulgarius (WGA)	$(\beta\text{-D-GlcNAc})_3$ > $(\beta\text{-D-GlcNAc})_2$ > β-D-GlcNAc
Ulex europaeus (UEA-I)	α-L-Fuc
Ulex europaeus (UEA-II)	$(\beta\text{-D-GlcNAc})_2$ > β-D-GlcNAc

[a] Specificities of all lectins were obtained from E-Y Laboratories or from Goldstein and Hayes (12).

[b] Abbreviations are: Gal, galactose; GalNAc, N-acetylgalactosamine; Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine: Fuc, fucose; 2-O-Me-D-Fuc, 2-O-methylfucose.

TABLE 2. Sources of bacteria used.

Bacillus	Source
B. anthracis 11956, 14185	American Type Culture Collection
B. cereus 6464, 7064, 19637, 11778 E14579, 23260, 13472, 246	(ATCC)
B. mycoides 6462	
B. lentus 10840	
B. globisporus 23301	
B. cereus, B. mycoides, B. brevis, B. megaterium, B. licheniformis B. circulans, B. pumilus	Midwest Culture Service, Terre Haute, IN
B. anthracis V-770, ATCC 4229, Colorado, KAN7322 S. Africa 205, M36, Texas, Ames, Vollum 1B, Sterne	USAMRIID, Fort Detrick, MD culture collection
B. cereus T, 4915, 9620, 9634	
B. mycoides USAMRIID	
B. thuringiensis 4040, 4041, 4042-b 4045, 4055, 4065	
B. sphaericus 1593	Bacillus Genetic Stock Center, Columbus, Ohio
B. anthracis 1103	U. of Michigan Research Laboratory
B. amyloliquefaciens N	M. Courtney, U. of Rochester
B. megaterium KM ade Prt⁻	S. Graham, U. of Louisville
B. circulans 14175, 14176, 9500, 11033, 7049, 4513	R.E. Gordon, Waksman Institute
B. polymyxa	
B. coagulans	

TABLE 3. Interactions between lectins and Group I *Bacillus* species[a].

Organism	APA	GSA-I	RCA-I	RCA-II	SBA	ConA	WGA	BPA	HPA	SRA	LPA
B. anthracis 11966[b]	+	+	+	+	+	-	-	-	-	-	-
B. anthracis 14185	+	+	+	+	+	-	-	-	-	w	-
B. anthracis 4229	+	+	+	+	+	-	+	-	-	-	-
B. cereus 4915	-	-	-	-	-	-	+	-	-	-	-
B. cereus 11778	-	+	-	-	-	-	+	-	+	-	-
B. cereus E14578	-	+	-	-	-	-	-	-	+	-	-
B. cereus 246	-	-	-	-	-	-	-	-	+	-	-
B. cereus T	-	-	-	-	-	-	-	-	w	-	w
B. cereus 7064	-	+	+	+	-	-	-	-	-	-	-
B. cereus 23260	-	-	-	-	-	-	-	-	w	-	-
B. cereus 19637	-	-	-	-	-	-	-	-	-	-	-
B. mycoides MWC	+	+	+	+	w	-	-	-	+	-	-
B. mycoides USAMRIID	+	+	+	+	w	-	-	-	+	-	-
B. mycoides 6462	+	+	+	-	+	-	-	-	+	-	-
B. thuringiensis 4040	-	-	-	-	-	-	-	-	-	-	-

[a]Agglutinations were scored as + (positive), - (negative) or w (weak). Lectin concentrations were 100 µg/mL. Assays, except for LPA, were in PBS. LPA assays were in 50 mM tris(hydroxymethyl)amino methane, 10 mM Ca^{2+}, pH 7.4.

[b]*B. anthracis* strains V770, Sterne, Ames, Colorado, Texas, S. Africa 205, M36, UMRL, and KAN 7322 gave identical agglutination

profiles as strain 11966. When B. anthracis ATCC 11966 was grown in a medium supplemented with 0.5% sodium bicarbonate (10) washed suspensions contained capsular material as evidenced by the binding of fluorescein-conjugated rabbit anti-poly(D-glutamic acid) antibody. These cells were similarly reactive with the lectins as the unencapsulated cells. B. cereus 6464, 9634, and 13472, as well as B. thuringiensis strains 4041, 4042-B, 4045, 4055 and 4060 were unreactive with any of the lectins.

[c] Lectins failing to agglutinate any of the bacteria included GSA-II, PNA, PEA, MPA, DBA, PHA-E, HAA, SJA, UEA-I, UEA-II, RPA, LBA, and Lotus A.

TABLE 4. Interactions between lectins and Art Groups II-VI with lectins—.

Organism	LECTIN APA	GSA-I	RCA-I	RCA-II	SBA	ConA	WGA	HPA	SRA	STA	LPA
Group II											
B. brevis[b]	-	-	-	-	-	-	-	-	-	-	-
B. lentus	-	-	-	-	-	-	-	-	-	-	-
Group III											
B. sphaericus	-	-	-	-	-	+	-	-	-	+	-
B. globisporus	-	-	-	-	-	-	-	-	-	-	-
Group IV											
B. subtilis 168	-	-	-	-	-	+	-	-	-	-	w
B. subtilis W23	-	-	-	-	-	-	-	-	-	-	w
B. amyloliquefaciens N	-	-	-	-	-	+	-	-	-	-	-
B. licheniformis	-	-	-	-	-	-	-	-	-	-	-
B. megaterium KM	-	-	-	-	-	-	-	-	-	-	-
B. pumilus	-	-	-	-	-	-	-	-	-	-	-
Group V											
B. circulans 7049	-	-	-	-	-	-	-	-	-	-	-
B. polymyxa	-	-	-	-	-	-	-	-	-	-	-
Group VI											
B. coagulans	-	-	-	-	-	-	-	-	-	-	-

[a]Conditions and interpretations were given in TABLE 3.

[b]Other bacteria failing to react included B. circulans strains 14175, 14176, 9500, 11033, 4513, and B. megaterium ade Prt⁻. Additional unreactive lectins were indicated in TABLE 3.

TABLE 5. *Bacillus* spores and lectin agglutination tests[a].

Spores	LECTIN													
	APA	GSA-I	RCA-I	RCA-II	SBA	ConA	WGA	HPA	SRA	RPA	HAA	GSA-II	UEA-II	MPA
B. anthracis 11966[b]	+	+	+	+	+	-	-	-	-	-	-	-	-	-
B. anthracis 14185	+	+	+	+	+	-	-	-	-	-	-	-	-	-
B. cereus T	-	-	-	-	-	-	w	+	-	-	w	w	-	-
B. cereus 6464	+	-	-	-	-	-	-	+	-	-	w	-	-	-
B. cereus 9634	-	-	-	-	-	-	-	+	-	-	w	-	-	-
B. cereus 23260	-	-	-	-	-	-	-	-	w	w	w	-	+	+
B. cereus E14579	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus 19637	-	-	-	-	-	+	-	+	-	-	+	-	-	-
B. cereus 246	-	-	-	-	-	w	-	-	-	-	-	-	-	-
B. mycoides 6462	w	-	w	w	-	-	-	+	-	-	-	-	-	-
B. mycoides MWC	w	w	w	w	+	-	-	+	-	-	-	-	-	-
B. subtilis 168	-	-	-	-	-	+	-	-	-	-	-	-	-	-

[a]Lectin concentrations and conditions for agglutinations were outlined in TABLE 3. Densities of spore suspensions were 0.5 absorbance units, 450 nm (1-cm).

[b]Other spore suspensions tested, but were unreactive, included *B. megaterium* KM, *B. licheniformis*, *B. subtilis* W23 and *B. thuringiensis* 4040.

Table 6. . Concentrations of lectins required for agglutination of vegetative cells and spores[a].

Organism	SBA	GSA-I	HPA
B. anthracis 14185	12.5 Neg	Neg Neg	Neg Neg
B. anthracis 11969	6.3 (50)	6.3 (50)	Neg Neg
B. anthracis	3.1 (12.5)	3.1 (25)	Neg Neg
B. anthracis 14185 (spores)	25 (50)	Neg Neg	Neg Neg
B. anthracis 11969 (spores)	25 (50)	Neg Neg	Neg Neg
B. mycoides 6462	100 (Neg)	100 (Neg)	3.1 (12.5)
B. mycoides MWC	25 (50)	12.5 (Neg)	6.3 (50)
B. mycoides USAMRIID	12.5 (50)	3.1 3.1	6.3 6.3
B. mycoides 6462 (spores)	Neg Neg	Neg Neg	25 (50)

[a] Agglutination reactions were described in TABLE 3. Values shown represent minimal concentrations of lectins required to elicit a positive agglutination reaction. Numbers in parentheses represent results obtained following the boiling of the cells or spores in PBS for 15 min.

TABLE 7. Interactions between *Bacillus* vegetative cells and spores with anti-*Bacillus subtilis gtaB* lipoteichoic acid[a].

Agglutinogen	Agglutinin titer[b]
B. anthracis 14185 cells	1:16
B. anthracis 14185 spores	1:16
B. anthracis 11966 cells	1:16
B. anthracis 11966 spores	1:16
B. anthracis MWC cells	1:16
B. anthracis MWC spores	1:16
B. mycoides 6462 cells	1:16
B. mycoides 6462 spores	1:16
B. mycoides MWC cells	1:16
B. mycoides MWC spores	1:16
B. subtilis 168 cells	1:64
B. subtilis 168 spores	1:64

[a]Antiserum was directed against a purified lipoteichoic acid from *B. subtilis gta* B290 (33). The rabbit antiserum was that used in another study ( ). The antiserum preparation (1.0 ml) was absorbed twice with 5 mg intact cell walls of *B. subtilis* W23. Assays were in 50 mM phosphate, 150 mM phosphate, 150 mM sodium chloride, pH 7.3, at room temperature.

[b]Values shown represent the highest dilutions giving rise to positive agglutinations. Cell (or spore) densities were adjusted to 0.5 absorbance units (1-cm) at 450 nm prior to use. Microtiter plates contained 50 µL agglutinogen and 50 µL antiserum.

TABLE 8. Chemical and enzymatic modification of the interaction between lectin and *Bacillus anthracis* and *Bacillus mycoides*[a].

Treatment[b]	*B. anthracis*		*B. mycoides*	
	SBA	HPA	SBA	HBA
None, control	6.25	Neg	100	1.6
0.1% SDS, 100°, 30 min	3.1	Neg	100	3.1
8 M urea, 2 hr	3.1	Neg	Neg	3.1
Succinic anhydride	12.5	Neg	100	3.1
Trypsin	6.3	Neg	Neg	3.1
Subtilisin	3.1	Neg	Neg	3.1
Lysozyme	6.25	Neg	200	3.1
Mutanolysin	12.5	Neg	Neg	3.1

[a]Conditions for interactions given in TABLE 3.

[b]Control cells were washed twice in, PBS. Cells treated with SDS were washed three times in water to remove the detergent, and then suspended in PBS. Succinic anhydride (5.0 mg/mL in acetonitrile) was added to a final concentration of 100 µg/mL cell suspension. The suspension was incubated 2 hr after which the cells were washed and suspended in PBS. Enzyme treatments (50 µg/mL final concentrations) were for 2 hr at 37° C. Cells were washed twice and suspended in PBS.

[c]Values shown are the minimal concentrations of lectins required for detectable agglutination. *B. anthracis* ATCC 11966 and *B. mycoides* ATCC 6462 were the bacteria used in the assays. Neg = no detectable agglutination.

LEGENDS FOR FIGURES

Figure 1. Binding of fluorescein-conjugated SBA to B. anthracis 11966.

Figure 2. Interaction between spores of B. anthracis 11966 and fluorescein-conjugated SBA.